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Fraaije, Marco W.; Mattevi, Andrea; Berkel, Willem J.H. van

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# Mercuration of vanillyl-alcohol oxidase from *Penicillium simplicissimum* generates inactive dimers

Marco W. Fraaije<sup>a</sup>, Andrea Mattevi<sup>b</sup>, Willem J.H. van Berkel<sup>a,\*</sup>

<sup>a</sup>Department of Biochemistry, Argicultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

<sup>b</sup>Department of Genetics and Microbiology, University of Pavia, via Abbiategrasso 207, 27100 Pavia, Italy

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**Abstract** Vanillyl-alcohol oxidase (EC 1.1.3.7) from *Penicillium simplicissimum* was modified with *p*-mercuribenzoate. One cysteine residue reacts rapidly without loss of enzyme activity. Three sulfhydryl groups then react in an 'all or none process' involving enzyme inactivation and dissociation of the octamer into dimers. The inactivation reaction is slowed down in the presence of the competitive inhibitor isoeugenol and fully reversible by treatment of the modified enzyme with dithiothreitol. Vanillyl-alcohol oxidase is more rapidly inactivated at low enzyme concentrations and protected from mercuration by antichaotropic salts. It is proposed that subunit dissociation accounts for the observed sensitivity of vanillyl-alcohol oxidase crystals towards mercury compounds.

**Key words:** Chemical modification; Flavoprotein; Heavy atom derivative; Quaternary structure; Vanillyl-alcohol oxidase; *Penicillium simplicissimum*

## 1. Introduction

Vanillyl-alcohol oxidase (VAO) from *Penicillium simplicissimum* catalyzes the oxidation of vanillyl-alcohol to vanillin with the simultaneous reduction of molecular oxygen to hydrogen peroxide [1]. The enzyme is a homooctamer of 0.5 MDA with each subunit containing 8 $\alpha$ -(*N*<sup>3</sup>-histidyl)-FAD as a covalently bound prosthetic group [1]. Based on the production of 4-hydroxycinnamyl alcohols from 4-allylphenols we have proposed that the reaction mechanism of VAO involves the formation of a *p*-quinone methide product intermediate [2]. To relate the chemical mechanism to protein function we have undertaken the X-ray analysis of VAO [3]. A major problem hampering structure determination proved to be the high sensitivity of the VAO crystals towards mercury and other heavy atom compounds. Therefore, we have studied the reactivity towards mercury of VAO in solution. In this paper, evidence is provided that mercuration of VAO involves the dissociation into dimers.

## 2. Materials and methods

### 2.1. Enzyme purification

Vanillyl-alcohol oxidase from *P. simplicissimum* (ATCC 90172) was purified as described [1,2].

### 2.2. Analytical methods

Absorption spectra were recorded at 25°C on an Aminco DW-2000 spectrophotometer. The concentration of VAO was determined spectrophotometrically using a molar absorption coefficient  $\epsilon_{430} = 12.5 \text{ mM}^{-1} \text{ cm}^{-1}$  for protein-bound FAD [1]. VAO substrates and competitive inhibitors have been described elsewhere [2]. VAO activity was

assayed at 25°C, pH 9.5, by following the conversion of vanillyl-alcohol to vanillin ( $\epsilon_{340} = 22.8 \text{ mM}^{-1} \text{ cm}^{-1}$ ) at 340 nm.

### 2.3. Chemical modification

4-Hydroxymercuribenzoate was obtained from Aldrich. Mercuration of VAO was performed at 25°C in 50 mM Hepes, pH 7.5. Incubation mixtures contained 1–10  $\mu\text{M}$  VAO and 100–300  $\mu\text{M}$  mercurial reagent. Aliquots were withdrawn from the incubation mixtures at intervals and assayed for residual enzyme activity. Reactivation of mercured VAO was achieved by the addition of 10 mM dithiothreitol. After a 2 h incubation at room temperature the activity of the enzyme was fully restored.

Incorporation of 4-hydroxymercuribenzoate into VAO was followed spectrophotometrically at 250 nm, using a molar difference absorption coefficient of  $7.6 \text{ mM}^{-1} \text{ cm}^{-1}$  to quantify the formation of mercaptide bonds [4]. Two-compartment cells were used with a total path length of 0.875 cm [5]. Both sample and reference cell contained 0.8 ml of 20  $\mu\text{M}$  enzyme in 50 mM Hepes, pH 7.5, in one compartment and 0.8 ml of 250  $\mu\text{M}$  4-hydroxymercuribenzoate in the same buffer in the other compartment. After recording a baseline, the reaction was initiated by mixing the solutions in the sample cell. The absorbance changes were followed by scanning spectra from 240 to 540 nm at a scan speed of 5 nm/s. At time intervals, aliquots were withdrawn from the sample cell and assayed for enzyme activity.

### 2.4. Analytical gel filtration

Analytical gel filtration at room temperature was performed on a Superdex 200 HR 10/30 column (Pharmacia ÅKTA system). Samples (50  $\mu\text{l}$ ) were eluted in 50 mM potassium phosphate, pH 7.0, containing 150 mM KCl, at a flow rate of 1.0 ml/min. Protein peaks were detected at 220 nm. Calibration proteins used were ferritin (450 kDa), catalase (232 kDa), yeast alcohol dehydrogenase (144 kDa), lipoamide dehydrogenase (100 kDa), *p*-hydroxybenzoate hydroxylase (88 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa) and cytochrome *c* (12.3 kDa). Analysis of the oligomeric state of VAO was done essentially as described in [6].

## 3. Results

### 3.1. Inactivation of VAO by 4-hydroxymercuribenzoate

Incubation of VAO with 4-hydroxymercuribenzoate led to complete loss of enzyme activity. The inactivation reaction followed pseudo-first-order kinetics and was slowed down in the presence of the competitive inhibitor isoeugenol (Fig. 1). The activity could be fully restored by treatment of the modified enzyme with 10 mM dithiothreitol. VAO was protected from inactivation by 4-hydroxymercuribenzoate in the presence of 0.5 M sodium chloride (Fig. 1). A similar protective effect was displayed by 0.2 M ammonium sulfate. Fig. 1 also shows that the rate of VAO inactivation by 4-hydroxymercuribenzoate increased at lower enzyme concentrations.

### 3.2. Incorporation of 4-hydroxymercuribenzoate into VAO

The incorporation of 4-hydroxymercuribenzoate into VAO was quantified by combined spectrophotometric and activity measurements. Fig. 2 shows that the time-dependent development of the difference spectrum involved absorbance changes

\*Corresponding author. Fax: (31) 317-484801.

E-mail: WILLEM.VANBERKEL@FAD.BC.WAU.NL

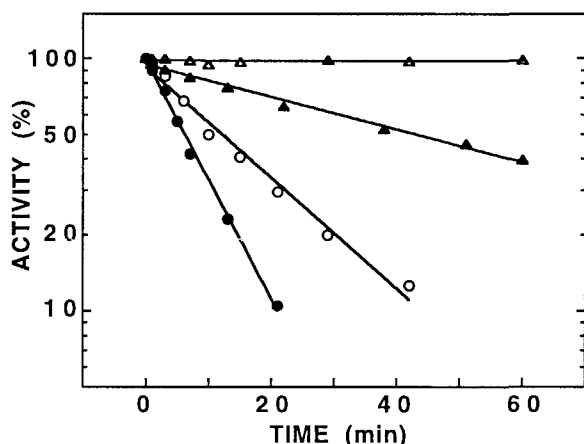


Fig. 1. Time-dependent inactivation of VAO by 4-hydroxymercuribenzoate. The temperature was 25°C. VAO in 50 mM Hepes, pH 7.5, was incubated with 200  $\mu$ M 4-hydroxymercuribenzoate: 1  $\mu$ M VAO (●); 10  $\mu$ M VAO (○); 10  $\mu$ M VAO in the presence of 200  $\mu$ M isoeugenol (▲); 10  $\mu$ M VAO in the presence of 0.5 M sodium chloride (△).

around 250 and 280 nm and in the flavin-absorbing region. Isosbestic points were observed at 305 and 450 nm. The kinetics at 280 nm and in the flavin-absorbing region were monophasic and parallel to those of the inactivation reaction (cf., Fig. 1). From the absorbance changes at 250 nm, indicative of the formation of mercaptide bonds [4], it can be concluded that about four cysteine residues are modified in VAO by 4-hydroxymercuribenzoate (inset, Fig. 2). One cysteine reacts rapidly without significant loss of enzyme activity. About three cysteines then react more slowly leading to complete inactivation (inset, Fig. 2). When the incorporation of 4-hydroxymercuribenzoate into VAO was studied in the presence of 0.2 M ammonium sulfate, nearly no absorbance changes occurred at 280 nm and in the flavin-absorbing region. About one cysteine residue was mercurated under these conditions as evidenced from the absorbance changes at 250 nm (not shown).

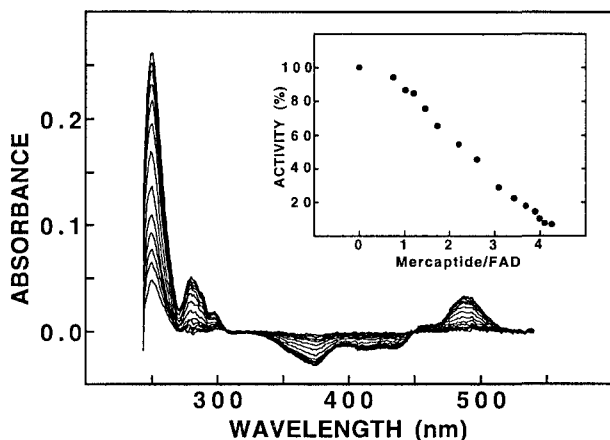


Fig. 2. Difference spectra observed during the inactivation of 10  $\mu$ M VAO by 125  $\mu$ M 4-hydroxymercuribenzoate in 50 mM Hepes, pH 7.5. Spectra are shown, recorded 1, 3, 5, 8, 12, 19, 31, 47, 61, 75, 90, 105 and 120 min after mixing. Inset: The formation of mercaptide bonds is plotted as a function of residual VAO activity.

### 3.3. Quaternary structure of native and 4-hydroxymercuribenzoate-treated VAO

Fig. 3A shows that treatment of VAO with 4-hydroxymercuribenzoate resulted in octamer dissociation. From running calibration proteins in parallel it was established that mercuriation of VAO involved the formation of dimers. The VAO dimers were inactive and the kinetics of subunit dissociation strongly resembled that of the inactivation reaction (cf., Fig. 1). Inactive dimers were also formed when VAO was treated with 4-hydroxymercuribenzoate in the presence of isoeugenol. Binding of isoeugenol, however, inhibited the rate of dimer formation and the fraction of octamers remaining paralleled the fraction of residual enzyme activity. The 4-hydroxymercuribenzoate-induced dissociation of VAO octamers was fully reversible as evidenced by treatment of the modified enzyme with dithiothreitol (not shown).

No subunit dissociation was observed when VAO was incubated with 4-hydroxymercuribenzoate in the presence of 0.2 M ammonium sulfate or 0.5 M sodium chloride. Analytical gel filtration revealed that this might be related to the stabilization of the octameric state. Fig. 3B shows that lowering the concentration of the native enzyme resulted in a considerable increase in the fraction of dimers. In agreement with urea unfolding experiments [10], the dimers were also active.

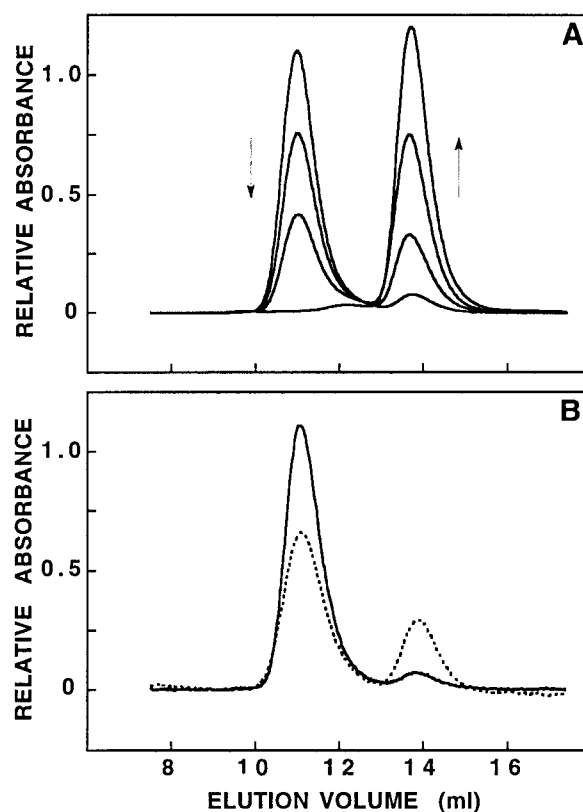


Fig. 3. Quaternary structure of native and mercurated VAO. A: 10  $\mu$ M VAO in 50 mM Hepes, pH 7.5, was incubated at 25°C with 200  $\mu$ M 4-hydroxymercuribenzoate. Superdex 200 HR elution profiles are shown, recorded 0, 10, 30 and 60 min after mixing. Arrows indicate the decrease and increase in the amount of octamers and dimers, respectively. B: Elution profiles of 1  $\mu$ M native VAO in 50 mM Hepes, pH 7.5, pre-incubated in the absence (broken line) or presence (continuous line) of 0.2 M ammonium sulfate.

shifted the octamer–dimer equilibrium in favor of the octameric state (Fig. 3B).

#### 4. Discussion

VAO represents a novel type of flavoprotein oxidase [2] and its X-ray structure is presently determined by the method of multiple isomorphous replacement [3]. In soaking experiments with mercury compounds we noticed that the VAO crystals are sensitive towards structural changes, influencing the crystal packing. In this paper evidence is provided that mercuriation of VAO involves quaternary structural changes causing the stabilization of dimers. Similar mercury-induced quaternary structural changes have been reported with other oligomeric enzymes like muscle phosphorylase [7], hemerythrin [8] and aspartate transcarbamoyltransferase [9]. The formation of VAO dimers is in line with results from urea unfolding [10] and the observation that the enzyme octamer has 422 symmetry [3].

Mercuriation of VAO resulted in complete inactivation and was accompanied by significant changes in the spectral properties of the covalently bound flavin. Because inactivation of VAO involved the modification of three cysteine residues it is at present not clear whether these spectral perturbations are directly related to subunit dissociation. Although the inhibitory effect of isoeugenol may point to the modification of a cysteine residue in the vicinity of the substrate binding site, the assignment of modification sites has to await the elucidation of the X-ray structure.

VAO was more rapidly inactivated at low enzyme concentrations and the enzyme was protected from mercuriation in

the presence of antichaotropic salts. This suggests that the mercuriation of VAO dimers shifts the octamer–dimer equilibrium of the native enzyme towards the dimeric state. In contrast to the native enzyme, octamerization of mercurated VAO was not stimulated in the presence of antichaotropic salts. This indicates that at least one of the cysteine residues is located near the dimer–dimer interface. Because VAO crystals grow in low salt [3] it is proposed that subunit dissociation accounts for the observed sensitivity of VAO crystals towards mercury compounds.

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